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The glycoprotein gene of Ebola virus contains a translational stop codon in the middle, thus preventing synthesis of full-length glycoprotein. Twenty percent of the mRNA isolated from Ebola virus-infected cells was shown to be edited, containing one additional nontemplate A in a stretch of seven consecutive A residues. Only the edited mRNA species encoded full-length glycoprotein, whereas the exact copies of the viral template coded for a smaller secreted glycoprotein. Expression of the glycoprotein by an *in vitro* transcription/translation system, by the vaccinia virus/T7 polymerase system, and by recombinant vaccinia virus revealed that full-length glycoprotein was synthesized not only when the edited glycoprotein gene (8A's) was used as a template for T7 and vaccinia virus polymerases, but also when the nonedited (genomic) glycoprotein gene was used. Analysis of mRNA produced by T7 and vaccinia virus polymerase from the 7A's construct revealed that 1–5% contained alterations at the same site that was also edited by the Ebola virus polymerase. Our data indicate that the editing site in the Ebola virus glycoprotein gene is recognized not only by Ebola virus polymerase but also by DNA-dependent RNA polymerases of different origin.

INTRODUCTION

Ebola virus (EBO), which together with Marburg virus (MBG) constitutes the family Filoviridae, is known to be an extremely pathogenic agent, causing a severe hemorrhagic disease in humans and monkeys with high mortality rates (Bowen *et al.,* 1977; Johnson *et al.,* 1977). EBO is an enveloped, single-stranded, negative-sense RNA virus (Kiley *et al.,* 1988; Peters *et al.,* 1994). EBO particles consist of at least seven structural proteins: the L protein (L), the glycoprotein (GP), the nucleoprotein (NP), and four other structural proteins, VP40, VP35, VP30, and VP24. The corresponding genes are located on the viral RNA in the following linear order: 3′-NP–VP35–VP40–GP–VP30–VP24–L-5′ (Sanchez *et al.,* 1993; Volchkov *et al.,* 1992, 1993; Feldmann *et al.,* 1993). All genes are transcribed into monocistronic polyadenylated messenger RNAs.

GP of EBO is highly glycosylated with an M_r of 125–140 kDa, when analyzed by sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis (PAGE) (Elliott *et al.,* 1985). Since GP is the only surface protein it is supposed to be responsible for receptor binding and membrane fusion during entry of the virus into host cells. It can be assumed that, in analogy to MBG, GP of EBO is present in the viral membrane as homotrimers that make up the virion surface spikes (Feldmann *et al.,* 1991). Recently it was shown (Volchkov *et al.,* 1992; Will *et al.,* 1992) that GP of filoviruses contains a stretch of 26 amino acids (aa) in the C-terminal region with a high degree of homology to an immunosuppressive motif found in the p15E-related glycoproteins of oncogenic retroviruses (Cianciolo *et al.,* 1985; Kadota *et al.,* 1991) which may contribute to the high pathogenicity of EBO and MBG.

There is evidence obtained from nucleotide sequence analyses suggesting that expression of GP involves interesting mechanisms at the transcriptional or translational level and that variations in the expression strategies of this gene exist. Sanchez and co-workers (1993) showed originally that full-length GP of the EBO Zaire was encoded by a single open reading frame (ORF). In contrast, a study carried out on the same strain by Volchkov and co-workers (1993) indicated that GP was expressed from two ORFs that are present in different frames. Two ORFs were also observed in a subsequent study of the American group on EBO subtypes Sudan and Reston as well as on virus of the subtype Zaire with a different passage history (Peters *et al.,* 1994). Furthermore, these authors obtained evidence that the primary gene product of the GP is not full-length GP, but a smaller nonstructural glycoprotein that is secreted from infected cells (sGP).

There are two different mechanisms by which a protein is expressed from different ORFs: mRNA editing as described for the P gene of paramyxoviruses, also belong-

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ing to the mononegavirales (Thomas *et al.,* 1988; Vidal *et al.,* 1990a; Cattaneo *et al.,* 1989; Paterson and Lamb, 1990) or ribosomal frame shifting as occurring with the gag–pol protein of retroviruses (Jacks *et al.,* 1988a,b; Wilson *et al.,* 1988).

In order to investigate the mechanism by which full-length GP is generated, GP-specific nucleotide sequences were determined at the genomic and at the mRNA level. Furthermore, transcription of GP mRNA and synthesis of the GP protein were studied in different expression systems. We show that synthesis of full-length GP proceeds via RNA editing. Editing was observed not only when GP was synthesized in EBO-infected cells, but also when the gene was transcribed using the vaccinia virus/T7 system and a recombinant vaccinia virus.

MATERIALS AND METHODS

Viruses and cell lines

The EBO subtype Zaire was received from the Institute of Virology (Sergiev Posad, Russia). This virus was once passaged in Macaca rhesus before use. EBO virus was cultured in Vero cells and purified from the tissue culture liquid as described previously (Volchkov *et al.,* 1992). The vaccinia virus strain WR and recombinant vaccinia virus vTF7-3 (kindly provided by Dr. B. Moss, Bethesda, MD) were propagated in E6 cells, a cloned cell line of Vero cells (ATCC CRL 1586). The recombinant vaccinia viruses were propagated in CV-1 cells as described by Mackett *et al.* (1986). RK13, CV-1, HeLa, and TK-143 cell lines were maintained in Dulbecco's medium containing 10% FCS (fetal bovine serum, GIBCO, Germany).

Molecular cloning and sequencing of viral RNA

The construction and identification of cDNA clones of EBO have been described previously (Volchkov *et al.,* 1992). The nucleotide sequence of the GP gene was determined by sequencing the inserts of the plasmids pEB137, pEB102, and pEB67. DNA sequence of the GP gene was determined for both strands. Sequence analysis was performed according to Maxam and Gilbert (1980). The sequence of the EBO GP gene strain Zaire had been deposited at GenBank under Accession No. U31033.

Genomic RNA of EBO was isolated from purified virus by centrifugation on cesium chloride gradients as described previously (Volchkov *et al.,* 1992). This RNA was used for the construction of a cDNA library using random primers and a reverse transcriptase from avian myeloblastosis virus. RNA–cDNA hybrids from the cDNA library were used for the amplification of the GP gene with synthetic oligonucleotide primers: N1 with the sequence 5′-GAAGGATCCTGTGGGGCAACAACACAATG (mRNA sense, nucleotides 114 to 142), supplied with a 5′-terminal *Bam*HI site; N2, 5′-AAAAAGCTTCTTTCCCTTGTCACTAAA (complementary to nucleotides 2492 to 2466, mRNA sense), supplied with a 5′-terminal *Hin*dIII site to facilitate cloning.

Isolation, cloning, and sequencing of EBO GP-specific mRNA

EBO GP-specific mRNAs were isolated from 7×10^7 Vero cells infected with EBO at a m.o.i. of 1–10 PFU per cell, 1 day p.i. Isolation was performed using the RNeasy Total RNA Kit (Qiagen, Germany) according to the instructions of the supplier.

For the first-strand cDNA synthesis, 10 μl RNA solution corresponding to 1.4×10^7 cells was used and reverse transcription was carried out with the primer N3 (oligo-$d(T)_{21}$), supplied with a 5′-terminal *Hin*dIII site. RNA thereafter was removed by incubation with 1 μg/μl RNAse at 37° for 30 min. Amplification of GP-specific mRNA via PCR was done using primers N1 and N3.

PCR was carried out in a total volume of 100 μl reaction mix containing 1–5 μg cDNA in 50 m*M* KCl, 10 m*M* Tris–HCl (pH 8.3), 2 m*M* $MgCl_2$, 0.2 m*M* of each deoxynucleotide, and 0.3 μM of each primer. The reaction mix was heated at 95° for 10 min and *Taq* polymerase (2.5 U/100 μl; "hot start PCR") was added. Thirty-five cycles of DNA amplification were performed. The cycling program was 94° for 1 min, 70° for 1 min, and 72° for 1 min. Thereafter, samples were incubated at 72° for 10 min. All components for PCR were obtained by Perkin–Elmer Cetus; the described protocol is a modified version of the RNA PCR protocol recommended by Perkin–Elmer Cetus. The products of PCR reaction were purified (QIAquick Spin PCR Purification Kit, Qiagen, Germany), and DNA was used directly for sequencing, for repeated PCR reactions, or for cloning in plasmid vectors. The nucleotide sequence of the region where ORF I and ORF II overlapped (Fig. 1A) was determined by the Sanger technique (Sanger *et al.,* 1977) utilizing the primers N4 and N5 with the sequence 5′-CGGACTCTGACCACTGAT (complementary to nucleotides 1108 to 1091) and 5′-TCGTGGCAGAGGGAGTGT (complementary to nucleotides 1412 to 1395), respectively.

Construction of recombinant plasmids

The entire ORF of the EBO GP gene was synthesized from purified vRNA or mRNA by the PCR technique as described above. In general the recombinant DNA procedures were run as described by Sambrook *et al.* (1989).

PCR products were digested with *Bam*HI and *Hin*dIII and ligated into the plasmid pGEM3Zf(+) that had been digested with *Bam*HI and *Hin*dIII. The vector contains a T7 phage RNA polymerase promoter which was used for synthesizing the EBO GP RNA with T7 polymerase *in vitro* and *in vivo* using the vaccinia virus/T7 polymerase expression system. The resulting recombinant plasmids containing the entire GP coding sequence of EBO vRNA were designated pGEM-mGP7 (7A's), the plasmid con-

taining the sequence of GP mRNA coding for the full-length GP (8A's) was designated pGEM-mGP8.

The GP-specific inserts were cut out from the plasmids pGEM-mGP7 and pGEM-mGP8 with *Bam*HI and *Hin*dIII, the ends of the fragments were filled up by Klenow fragment of DNA polymerase I and ligated into the *Sma*1 restriction site of the vector pSC11 (Promega, Madison, WI). The obtained recombinant plasmids, pSC-mGP7 and pSC-mGP8, were used for the generation of recombinant vaccinia viruses.

In vitro transcription/translation

For *in vitro* translation, a coupled transcription and translation kit (TNT, Promega) was used. GP mRNA was transcribed from 5 μg of recombinant plasmids pGEM-mGP7 or pGEM-mGP8 according to the instructions of the supplier and translated directly with a rabbit reticulocyte lysate system. One-fifth volume of each reaction was analyzed by 10% SDS–PAGE and autoradiography.

EBO GP-specific mRNAs transcribed by T7 RNA polymerase from plasmids pGEM-mGP7 or pGEM-mGP8 were purified as described above (RNeasy Kit, Qiagen) followed by digestion of plasmid DNA with RQ1 RNase-free DNase (Promega) at a concentration 2 units/μg plasmid DNA for 30 min at 37°.

Primer extension method

The primer extension procedures were carried out essentially as described by Pelet *et al.* (1991), except that 20 μg of purified RNA (RNeasy Kit Qiagen) was used in the presence of 0.5 m*M* of each dNTP and 0.8 m*M* ddGTP. The control reaction included the same amount of RNA from uninfected cells. The following primers were used: N6 5′-GAATTTTTCTAGTGAGG (complementary to nucleotides 1043 to 1027) and as a negative control N7 5′-TACTCTGTCTCATTGGTT (complementary to nucleotides 834 to 817). The 3′ end of N6 is located just downstream of the editing site.

Transient expression with T7 polymerase/vaccinia virus

For transient expression of GP, 1×10^6 HeLa cells were infected with vTF7-3 at an m.o.i. of 10 PFU/cell. At 1 hr p.i., the virus inoculum was replaced by the transfection inoculum containing 5 μg recombinant plasmids pGEM-mGP7 or pGEM-mGP8 and 5 μl lipofectin (BRL) (Felgner *et al.,* 1987). At 16 to 18 hr after transfection, medium and cells were harvested separately and analyzed for expression of GP.

EBO GP-specific mRNAs from transfected HeLa or RK13 cells were purified as described above (RNeasy Kit, Qiagen) followed by digestion of DNA with RQ1 RNase-free DNase (Promega) for 30 min at 37°, 2 units/μg plasmid DNA.

Construction of recombinant vaccinia viruses and isolation of RNA

Recombinant vaccinia viruses were generated by homologous recombination between tk regions in the recombinant plasmids pSC-mGP7 (7A's at the editing site) or pSC-mGP8 (8A's) and the genomic DNA of vaccinia virus (WR strain) as described by Chakrabarti *et al.* (1985) using the lipofectin transfection method (Felgner *et al.,* 1987). Recombinant viruses were plaque-purified one time on TK-143 cells and four times on CV-1 cells using β-galactosidase-positive plaques for selection. Recombinant vaccinia viruses which were derived from pSC-mGP7 were designated vSCGP7 and those derived from plasmid pSC-mGP8 were designated vSCGP8. Expression of GP was performed by infecting 1×10^6 HeLa cells with vSCGP7 or vSCGP8 at an m.o.i. of 10 PFU per cell. After 16 to 18 hr p.i. infected HeLa cells and medium were harvested separately and analyzed for expression of GP.

EBO GP mRNAs from infected HeLa or RK13 cells were purified as described above.

Immunoblot analyses

Lysates from 1.4×10^5 infected cells (RK13 or HeLa) were separated on 10% SDS–PAGE as described by Laemmli (1970) and blotted onto PVDF membranes (Millipore) by the semidry technique. When secreted sGP was analyzed, the supernatant from 1×10^6 infected cells was harvested (2 ml), and 20 μl was loaded onto the gel. Immunodetection was performed using either a mouse anti-EBO serum (1:1000) or a horse anti-EBO serum (1:2000) (kindly provided by Dr. I. Borisevich, Sergiev Posad, Russia) and a rabbit anti-mouse or rabbit anti-horse secondary antibody coupled to horseradish peroxidase (1:5000). Bound secondary antibody was detected by ECL technique (Amersham).

RESULTS

Molecular cloning and sequencing of the entire GP gene from vRNA

The sequence of the GP gene of EBO subtype Zaire has been determined as described under Materials and Methods and has been deposited at GenBank (Accession No. U31033; Volchkov *et al.,* 1993). As already pointed out, the sequence was identical to that published by Sanchez *et al.* (1993), with one important difference. At position 1019 to 1025 we found seven instead of eight consecutive A's (mRNA sense). The amino acid sequence deduced from the nucleotide sequence showed two large ORFs present in different frames. The first ORF extends from the ATG codon at position 2 or, more likely, from ATG at position 142 (Sanchez *et al.,* 1993) to the TAA at position 1232. This ORF is capable to encode a protein of 410 or 364 aa, respectively. A second ORF consisting of 382 aa (ORFII) was found between nucleo-

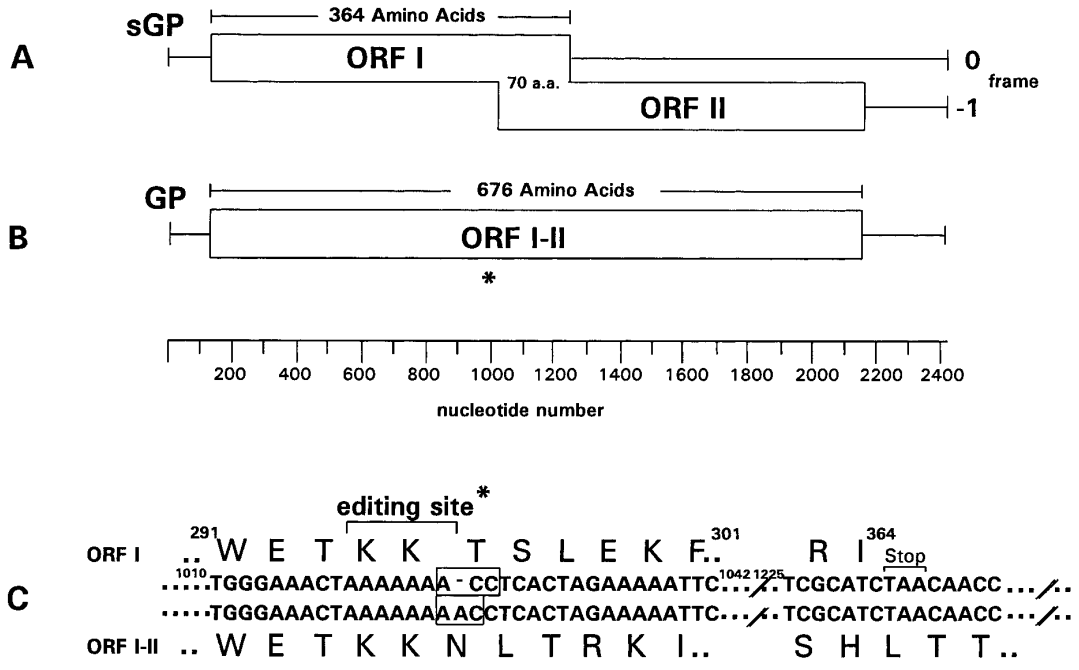

FIG. 1. Schematic representation of the EBO GP gene, its ORFs, and its mRNAs. (A) sGP represents the mRNA which is an exact copy of the genomic RNA, coding for the sGP protein. The two predicted overlapping ORFs in frame (0) and (−1) are shown as boxes. (B) GP represents the mRNA which is edited by insertion of one A residue coding for the full-length GP. The asterisk under the fusion ORF I–II indicates the position of the editing site. Nucleotide positions are numbered. (C) The nucleotide sequence and the deduced amino acid sequence of the EBO GP editing site without and with the A insertion. The point of transition from ORF I to ORF II is boxed.

tides 1021 to 2166 in (−)1 reading frame to the first ORF (Fig. 1A). None of these two ORFs was able to encode a protein of the size of GP found in virions (approx 125–140 kDa) (Elliott *et al.,* 1985). To exclude the possibility that the sequence difference between both studies was the result of a cloning artifact, multiple clones independently isolated have been analyzed. Forty cDNA clones containing the GP gene were sequenced, all of them showed only seven consecutive A's (mRNA sense) in the above-mentioned region. This observation indicates that the GP gene of the virus analyzed in our study contains a stretch of seven A's at position 1019–1025 (mRNA sense).

Molecular cloning and sequencing of the entire GP gene from mRNAs

mRNA was isolated from EBO strain Zaire-infected Vero cells and transcribed into cDNA, and GP-specific cDNA was amplified by PCR. Resulting PCR fragments were cloned into the vector pGEM3Zf(+), and recombinant plasmids were analyzed by enzymatic sequencing (Sanger *et al.,* 1977). The sequences of the 40 analyzed clones differed from each other only by the number of A residues present between nucleotides 1018 and 1026 (mRNA sense, mRNA numeration). In seven cases eight A residues could be identified, whereas all others contained seven A's representing exact copies of the vRNA template.

These data indicated that approx 20% of GP-specific mRNA's in EBO-infected cells contained one nontemplate A residue at this specific site. Only these 20% of GP mRNAs are able to encode full-length GP of 676 aa because insertion of the additional A results in a frameshift of ORF I to ORF II. Eighty percent of the analyzed mRNA clones encode presumably the nonstructural second glycoprotein (sGP). As indicated by the nucleotide sequence sGP is identical to the N-terminal 294 aa of GP and contains additional 70 aa which are encoded by the end of ORF I. These aa do not occur in GP because the additional A shifted the reading frame to ORF II before ORFI is closed. The predicted aa sequence shows also that sGP does not contain the GP membrane anchor (Volchkov *et al.,* 1992; Sanchez *et al.,* 1993). However, since it has the same N-terminal signal sequence as full-length GP, sGP should be translocated into the ER (Sanchez *et al.,* 1993). This concept is compatible with the observation that sGP is secreted into the medium of infected cells (Peters *et al.,* 1994).

For further investigation, the GP sequence was cloned into several expression plasmid vectors containing either 7A's (pGEM-mGP7, pSC-mGP7) or 8A's (pGEM-mGP8, pSC-mGP8) at the editing site.

In vitro translation of GP RNA transcripts synthesized using T7 RNA polymerase

In vitro translation of pGEM-mGP8 (8A's, corresponding to the edited mRNA species) resulted in the formation of three proteins: a major 75-kDa protein, a second poly-

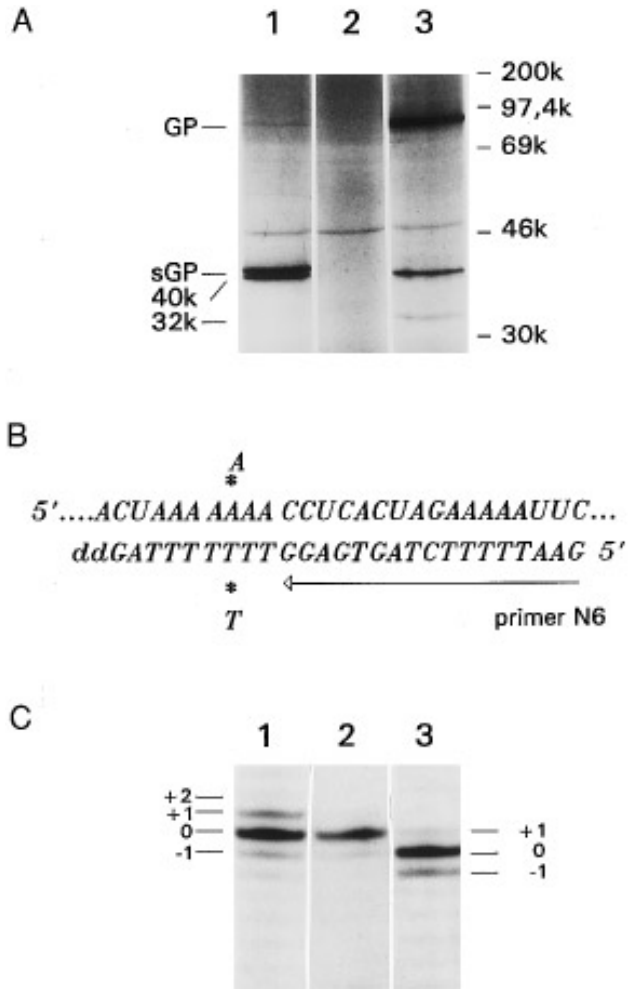

FIG. 2. *In vitro* analysis of protein and mRNA of EBO GP. (A) Expression of EBO GP from *in vitro* transcribed GP mRNA. The plasmids pGEM-mGP7 (7A's) and pGEM-mGP8 (8A's) were constructed by inserting cDNA from EBO GP mRNA into plasmid pGEM3Zf(+). Plasmids were transcribed *in vitro* with T7 RNA polymerase and translated by a rabbit reticulocyte lysate. Lane 1, plasmid pGEM-mGP7 (7A's); lane 2, pGEM3Zf(+) as a control; lane 3, pGEM-mGP8 (8A's). The positions of the GP, sGP, GP-specific proteins 40k or 32k, and molecular mass standards are indicated. (B) Limited primer extension analysis of the GP mRNA synthesized by T7 RNA polymerase. The mRNA sequence of GP gene (position 1016–1042) is depicted, and the insertion site is indicated with an asterisk. The underlined primer is extended by RT in the presence of ddGTP which terminates extension at the first C residue upstream of the insertion site. (C) Two different regions of GP mRNA were analyzed by primer extension: editing site of pGEM-mGP8 (lane 1), of pGEM-mGP7 (lane 3) with primer N6 and a region with no history for editing (nt 808–836) (lane 2) with primer N7. The lines on the left and right refer to the various primer extension products.

peptide with approx 40 kDa, and a third 32-kDa protein (Fig. 2A). Translation of pGEM-mGP7 (7A's) resulted in a major polypeptide band migrating at approx 42 kDa (sGP), a polypeptide with approx 40 kDa, and an additional longer protein with a M_r of approx 75 kDa corresponding to the unglycosilated EBO GP. The amount of GP observed after translation of pGEM-mGP7 was approx 5% compared to the amount of GP expressed by pGEM-mGP8. The 40-kDa protein detected after translation of both plasmids is presumably the product of the ORF II starting with the internal ATG codon at position 1186, since all other ATG codons will not give rise to a protein of the detected size. The occurrence of the additional GP-specific proteins during translation of pGEM-mGP7 (75 kDa) and pGEM-mGP8 (32 kDa) was unexpected and suggested either RNA editing as shown for the EBO mRNA in infected cells (see above) or translational frameshifting.

In order to prove the first hypothesis a primer extension method was used as described by Pelet *et al.* (1991). Extension of the primer across the editing site of recombinant RNA (cRNA) yielded a major band at position 0 corresponding to the unaltered RNA (ddGTP was included in the reverse transcription reaction so that synthesis would terminate at the first C upstream of the insertion site, Fig. 2B). An additional weaker signal occurred that represented GP cRNA elongated by one nucleotide, and this was obviously the result of editing by the T7 polymerase. As shown in Fig. 2C (lane 2), primer extension analysis of a region with no history for editing events yielded only a single band at the expected position. It is therefore clear that insertion of one nucleotide was the predominant editing event. An insertion of two nucleotides was also detectable (data not shown). It was not possible to unambiguously identify cRNA species with deletions because a ladder of interfering shorter bands were detected also in negative controls. These bands are likely to be degradation products of the primer, since shorter minor bands were also detected when the primers were electrophoresed without extension (data not shown). These results indicate that the 75-kDa protein found after *in vitro* transcription/translation of pGEM-mGP7 (Fig. 2A, lane 1) is derived from a mRNA species with an additional nucleotide at the editing site leading to an ORF which encodes full-length GP. In analogy, the 32-kDa protein observed after *in vitro* transcription/translation of pGEM-mGP8 must be explained by a mRNA with an extra nucleotide in the region of the editing site (Fig. 2A, lane 3). This insertion leads to the termination of the ORF I just downstream of the editing site (stop codon at position 1073). The expected size of the protein corresponded to the observed 32-kDa band.

Expression of GP gene with the vaccinia/T7 polymerase expression system

The expression of the EBO GP gene was then examined using the vaccinia virus/T7 RNA polymerase expression system (Fuerst *et al.,* 1986). The plasmids pGEM-mGP7 and pGEM-mGP8, containing the GP gene downstream of the T7 RNA polymerase promoter in the pGEM3Zf(+) vector, were transfected into HeLa or rabbit kidney (RK13) cells which had previously been infected with vTF7-3 (Lefkowitz *et al.,* 1990). Transfected cells and supernatants were harvested 16–18 hr posttransfection. Proteins were analyzed by SDS–PAGE and Western blot.

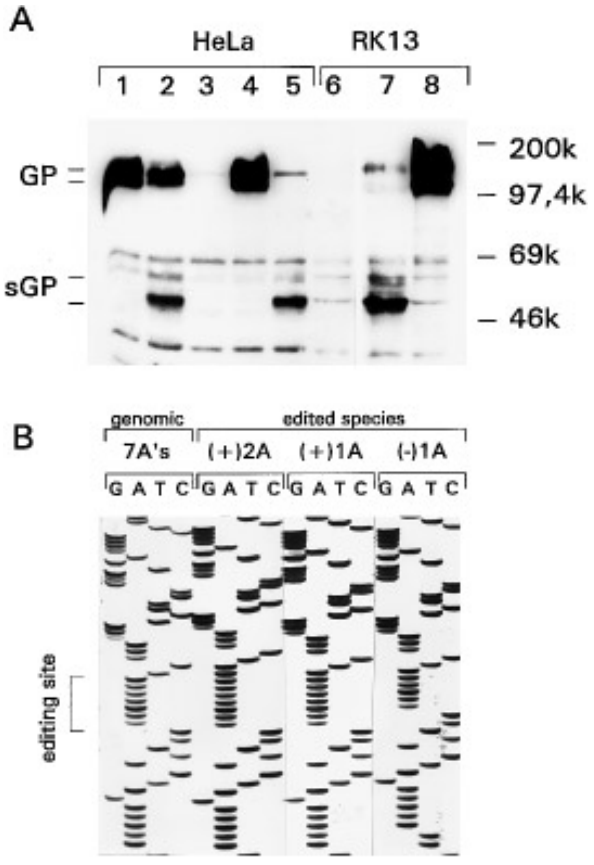

FIG. 3. Analysis of *in vivo* expressed GP. (A) Western blot analysis. Cell lysates of HeLa or RK13 cells either transfected by pGEM-mGP7 or pGEM-mGP8 or infected by recombinant vaccinia viruses vSCGP7 or vSCGP8 were separated by SDS–PAGE and blotted onto PVDF membranes prior to probing with anti-EBO antiserum. Lanes 1–5, HeLa cells; lanes 6–8, RK13 cells. Lane 1, pGEM-mGP8 transfected; lane 2, pGEM-mGP7 transfected; lane 3, control (vTF7-3 infected); lane 4, vSCGP8 infected, lane 5, vSCGP7 infected; lane 6, control (vTF7-3 infected); lane 7, vSCGP7 infected; lane 8, vSCGP8 infected. The positions of GP, sGP, and molecular mass standards are indicated. (B) Sequence of GP-specific mRNA clones obtained from vaccinia virus/T7 expression system. The nucleotide sequence of GP cDNA clones derived from different mRNA species transcribed in vaccinia virus/T7 expression system in the region of nucleotides 996–1039 are shown to illustrate the 6, 7, 8, or 9 A residues in the EBO GP editing site. The A's at positions 1019–1024 are marked.

Expression of pGEM-mGP8 resulted in two EBO-specific proteins migrating at approx 125 and 110 kDa in HeLa cells as shown in Fig. 3, lane 1, or 140 and 110 kDa in RK13 cells (data not shown). As shown below, endo H digestion revealed that the 110- and the 125- to 140-kDa species represented the high mannose and mature forms of GP, respectively.

When pGEM-mGP7 was used, the main GP-specific expression product migrated at approx 50 kDa, but additionally both forms of full-length GP were present, which were indistinguishable from pGEM-mGP8-derived GP. To determine whether full-length GP produced under these conditions was translated from edited mRNA, RNA was isolated from vTF7-3-infected and pGEM-mGP7-transfected HeLa cells, and 80 GP-specific cDNA clones were sequenced. These clones differed only in the amount of A residues at the editing site: two were found with an additional A (corresponding to mRNA2), one contained nine A's, two contained only six A's, whereas all others were found to be complementary copies of the plasmid pGEM-mGP7 (Fig. 3B). This result indicated that, indeed, edited mRNA was present which allowed the synthesis of full-length GP.

The amount of full-length GP was 5–10% when compared to the expression of GP by pGEM-mGP8. The GP expression products obtained with the vaccinia/T7 polymerase system differed in size from GP transcribed *in vitro.* Whereas the longer expression product of pGEM-mGP8 migrated at 75 kDa when synthesized *in vitro* (Fig. 2A, lane 3), expression of the full-length GP with the vaccinia/T7 polymerase system resulted in the described two forms migrating at 110 and 125–140 kDa. Likewise, sGP had a size of 42 kDa after *in vitro* translation and approx 50 kDa in the vaccinia/T7 system. These differences were due to glycosylation of the proteins as shown below (Fig. 4).

sGP could not be detected in purified EBO virions (Elliott *et al.,* 1985; Chepurnov *et al.,* 1994), indicating that it is a nonstructural secreted glycoprotein (Peters *et al.,* 1994).

Expression of GP by recombinant vaccinia viruses

Since T7 DNA-dependent RNA polymerase was shown to edit the EBO-specific cDNA constructs, it was of interest to investigate another RNA polymerase for its capacity to edit the GP gene. For this purpose recombinant vaccinia viruses were constructed containing either GP mRNA1 (7A's) or mRNA2 (8A's). HeLa cells and RK13 cells were infected with the resulting recombinant viruses designated vSCGP7 and vSCGP8 and analyzed for expression of GP gene products by immunoblot analysis (Figs. 3A, 4A and 4B). Two EBO-specific bands were detected in the cell lysates infected by vSCGP8 (Fig. 3A, lanes 4 and 8). M_r of both GP species coincided with those obtained by transient expression of pGEM-mGP8 (Fig. 3A, lane 1). However, the amount of GP was higher after expression from recombinant vaccinia viruses. When HeLa or RK13 cells were infected with vSCGP7 (genomic sequence), the ratio between sGP and full-length GP was different from that obtained in the vaccinia/T7 system. Whereas the amount of full-length GP expressed from vSCGP7 was decreased, the expression rate of secreted sGP was higher compared to the vaccinia/T7 system (data not shown). Since the experiments were performed in the same cells (HeLa or RK13), the only difference in the synthesis of GP was at the level of transcription of mRNA that was either catalyzed by T7 RNA polymerase or by vaccinia virus polymerase. Sequencing of 80 cDNA clones obtained from mRNA of vSCGP7-infected cells showed that one clone contained

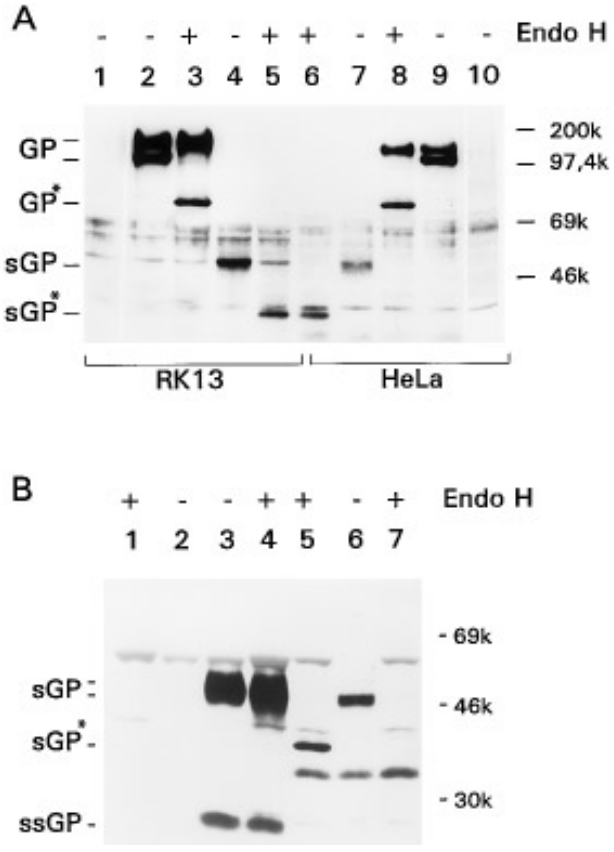

FIG. 4. Endo H digestion analysis of GP expressed in HeLa or RK13 cells. Cells were infected with vSCGP7, vSCGP8, or with vTF7-3. At 18 hr p.i. supernatants or cells were lyzed and subjected to glycosidase treatment. Digested samples were separated by SDS–PAGE and blotted onto PVDF membranes. Detection of GP-specific proteins was performed with a horse anti-EBO antiserum. GP-specific proteins digested by Endo H are marked by asterisks. The position of the GP, sGP, super sGP (ssGP), and molecular mass standards are indicated. (A) Analysis of GP expression in RK13 (lanes 1–5) or HeLa cells (lanes 6–10). Lane 1, control (vTF7-3 infected); lane 2, vSCGP8 infected; lane 3, vSCGP8-infected cells after digestion with endo H; lane 4, vSCGP7-infected cells; lane 5, vSCGP7-infected cells after digestion with endo H; lane 6, vSCGP7-infected cells after digestion with endo H; lane 7, vSCGP7-infected cells; lane 8, vSCGP8-infected cells after digestion with endo H; lane 9, vSCGP8-infected cells; lane 10, control (vTF7-3-infected cells). (B) Analysis of GP-specific proteins in the supernatant of HeLa cells. Lane 1, supernatant of vTF7-3-infected cells after digestion with endo H; lane 2, supernatant of vTF7-3-infected cells; lane 3, supernatant of vSCGP7-infected cells; lane 4, supernatant of vSCGP7-infected cells after digestion with endo H; lane 5, vSCGP7-infected HeLa cells after digestion with endo H; lane 6, vSCGP7-infected HeLa cells; lane 7, vTF7-3-infected cells after digestion with endo H.

an additional A residue, thus again supporting the view that synthesis of full length GP is caused by editing of the 7A's stretch.

To investigate the nature of the two GP-specific proteins (110 and 125–140 kDa) found after expression from recombinant vaccinia viruses, infected cell lysates were digested with endoglycosidase H (endo H). Figure 4A (lanes 3 and 8) shows that the 110-kDa species shifted to 75 kDa corresponding to the unglycosylated GP. The 125- to 140-kDa forms were resistant to endo H digestion indicating that these species represented the mature forms of GP with complex type N-linked oligosaccharides.

The size of the unglycosylated form of GP and the size of the high mannose form were independent from the cell type used for expression. However, the mature GP expressed in RK13 cells migrated at 140 kDa in SDS–PAGE, whereas GP in HeLa cells had a size of 125 kDa. This difference is probably due to variations in complex type N-glycosylation of the two cell lines. The 140-kDa form detected in RK13 cells comigrated with GP incorporated into the virions.

Supernatants of vSCGP7-infected cells were shown to contain sGP migrating as a diffuse band of approx 50–55 kDa (Fig. 4B, lane 3). Intracellular sGP migrated as a band of 50 kDa. Endo H digestion of lysates and supernatants of cells infected with vSCGP7 revealed that the secreted sGP was resistant to endo H, whereas the smaller intracellular form was converted to a 40-kDa protein (Fig. 4B, lanes 3–6). Only trace amounts of the mature sGP were detected intracellularly (Fig. 3A, lanes 2, 5, and 7). As can be seen in Fig. 4B, (lanes 3 and 6), the major part of sGP synthesized in cells accumulates in the culture medium. Quantitative analysis showed that the ratio between secreted sGP and intracellular form of sGP is approximately 25:1. These data indicate that sGP is rapidly secreted after processing to the complex form. In addition to sGP, another GP-specific protein could be detected in the supernatant of vSCGP7 migrating at an M_r of approx 28 kDa (Fig. 4B, ssGP). This protein is possibly the translation product of GP-specific mRNAs with a deletion of one or an insertion of two A residues at the editing site, that were detected when the transcripts of the 7A's constract were sequenced. Both mutations would lead to a termination of translation at position 1073 and therefore to the synthesis of proteins with the detected size (see above). However, it is also possible that this protein is the translation product of a mRNA that arises when the GP editing site is used as a stop signal by the vaccinia virus polymerase (Moss, 1990). Further studies are needed to examine whether ssGP occurs as a second nonstructural protein in EBO-infected cells or whether it is observed only in vaccinia virus expression systems.

DISCUSSION

GP (M_r 125–140 kDa) is the only surface protein of EBO. It is integrated into the viral membrane from which it can be removed by bromelain treatment (Kiley *et al.,* 1988). Nucleotide sequence analysis performed on EBO strain Zaire revealed the presence of a stop codon in the middle of the GP gene (Volchkov *et al.,* 1993). This observation has now been confirmed on a total of 43 cDNA clones, none of which showed a single ORF capable to encode a protein of the size of GP. From these results it became clear that expression of EBO GP in-

volves either translational frame shifting or RNA editing at the level of transcription.

When GP-specific mRNA of EBO-infected cells was analyzed, 80% of the sequenced cDNA clones were found to be exact copies of the viral genome. However, 20% contained eight instead of seven A residues at positions 1019 to 1026 resulting in a frameshift that allows the synthesis of full-length GP. These observations indicate that synthesis of full-length GP involves editing by the viral polymerase. This concept is in agreement with the latest work of Sanchez and co-workers. Although in their original report on the genome of the Mayinga strain of EBO GP was found to be encoded by a single ORF (Sanchez *et al.,* 1993), extension of their studies to subtypes Reston and Sudan and recently to Zaire virus with a different passage history revealed that GP is expressed by transcriptional editing from two ORFs (Peters *et al.,* 1994, Sanchez *et al.,* 1994). Thus, editing of the GP gene appears to be a common trait of EBO, whereas it has so far not been observed with MBG.

The observation that, depending on the propagation history of the virus, the GP gene of the Zaire subtype contains either 7U's or 8U's at the editing site is of considerable interest. It remains to be seen whether these variants arise from each other by mutation or whether they segregate from a mixed population during passage in cell culture or in experimental animals. Obviously, the differences in the replication strategies of both variants may have important consequences for virus spread, course of infection, and pathogenicity. Further studies are needed to throw light on these problems.

To investigate the expression products of both types of EBO GP mRNA, expression of GP was studied in several expression systems, using two cDNA constructs: one with 7A's at the proposed editing site, corresponding to the genomic sequence, the other with 8A's at the same site, representing the edited mRNA species. Translation of the 8A's species with an *in vitro* system using T7 DNA-dependent RNA polymerase for transcription revealed the expected unglycosylated full-length GP at a size of approx 75 kDa (Fig. 2A, lane 3). Expression of the 7A's construct led to sGP (42 kDa) corresponding to ORFI. Surprisingly, also full-length GP was detected after *in vitro* transcription/translation of the 7A's construct.

Similar results were obtained when GP was synthesized by the vaccinia virus/T7 system, suggesting that the T7 RNA polymerase, like EBO polymerase in virus-infected cells, was able to edit the GP gene. mRNA synthesized by the T7 polymerase was analyzed by sequencing and primer extension. Both methods detected edited mRNA species. Two mRNA clones of 80 contained 8A's at the editing site allowing the synthesis of full-length GP. In addition, 6A's and 9A's species could be detected, suggesting that editing may not lead in every case to a (+)1 mutant mRNA but also provide (−)1 and (+)2 mutants (Fig. 3B). Interestingly, Jacques *et al.* (1994) showed that RNA editing in the P gene of paramyxoviruses may also result in deletions. Deletions were mainly found when seven or more identical nucleotides (C) occurred in the template.

To confirm the data derived from *in vitro* transcription/translation, GP was transcribed by vaccinia virus RNA polymerase. Expression from recombinant vaccinia viruses revealed that full-length GP was also obtained when the 7A's construct was used. Since the amount of 8A's mRNA species transcribed from the 7A's virus was lower than that observed in the T7-driven system, it was not surprising that the amount of full-length GP was also decreased.

RNA editing has been described for a variety of paramyxoviruses, such as simian virus 5 (SV5, Thomas *et al.,* 1988), Sendai virus (Vidal *et al.,* 1990a), measles virus (Cattaneo *et al.,* 1989), mumps virus (Paterson and Lamb, 1990; Takeuchi *et al.,* 1990), and parainfluenza virus types 2 and 4 (Southern *et al.,* 1990; Ohgimoto *et al.,* 1990; Kondo *et al.,* 1990). With each of these viruses, editing has been shown to occur by the insertion of an additional G residue at the specific sequence 3′-UUU/CUCCC-5′ of the P gene. The P gene of paramyxoviruses is reported to be edited exclusively by viral RNA-dependent RNA polymerases (Horikami and Moyer, 1991; Pelet *et al.,* 1991; Matsuoka *et al.,* 1991; Vidal *et al.,* 1990a). In contrast, the data presented here show that the editing site of EBO GP is also recognized by DNA-dependent RNA polymerases. The mechanism by which editing of the GP gene occurs appears to be similar with RNA- and DNA-dependent RNA polymerases, since both types of enzymes insert exactly in the same region the same non-template nucleotide (A). The detected editing site in EBO genomic RNA (7U's) is different from the editing site found in the P gene of paramyxoviruses, rather resembling the polyadenylation site (transcription stop signal) of EBO mRNAs (3′-UAAUUCUUUUUU, genomic sense). The EBO editing site is also similar to the transcription stop signal of the vaccinia virus polymerase (3′-UUUUUNU) (Moss, 1990). Presumably, temporary pausing of the viral RNA polymerase and both investigated DNA-dependent RNA polymerases at the editing site of the EBO GP gene enables the transcription complex to slip backward or forward on the vRNA template before the next nucleotide is incorporated. This mechanism was described for editing of the P gene of paramyxoviruses (Vidal *et al.,* 1990b) and obviously could be used in editing of EBO GP. In this case the similarity with polyadenylation sites may at least partly explain the relatively broad spectrum of RNA polymerases recognizing the editing site of EBO GP.

Our data indicate that editing of the GP gene is most likely the main source of the full-length GP in EBO-infected cells and in cells which were transfected with pGEM-mGP7 or infected by vSCGP7. However, it is important to note that the proposed EBO GP editing site is also well suited for a ribosomal frame shifting as shown for mouse mammary tumor virus, simian T-cell leukemia

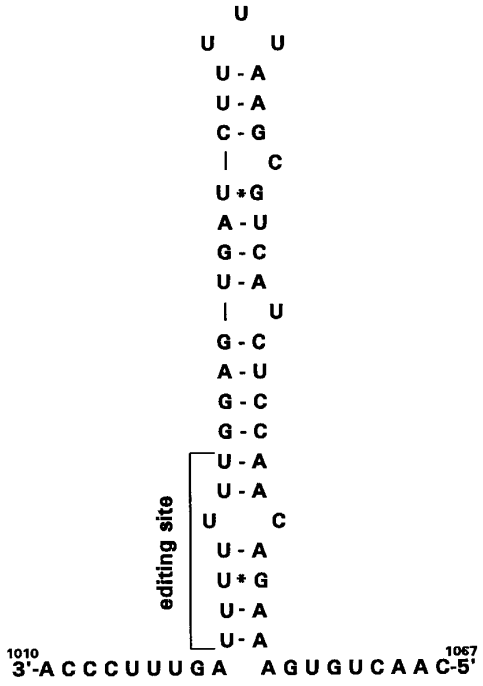

FIG. 5. RNA secondary structure predictions. The EBO RNA sequence from nucleotides 1010–1068 of the GP gene was examined for regions with strong secondary structure using the Heidelberg UNIX Sequence Analysis Resources (HUSAR), release 3.0, program FOLDRNA. The stem–loop structure shown has an energy of $\Delta G = -12.4$ kcal/mol. The seven U residues of the editing site at nucleotides 1019–1025 are indicated.

virus (Jacks *et al.,* 1987, Hatfield *et al.,* 1992), and astrovirus (Lewis and Matsui, 1995). To date we cannot rule out completely that ribosomal frame shifting occurs also during EBO GP synthesis in expression systems and in EBO-infected cells, but the evidence presented here shows clearly that RNA editing is sufficient to explain the existence of all detected GP-specific proteins in the investigated expression systems. Finally, the observed differences between the vaccinia virus/T7 and the recombinant vaccinia virus systems concerning the level of full-length GP synthesis can only be explained by differences in the level of editing activity of the used RNA polymerases.

The efficiency of editing is higher with the EBO polymerase than with the investigated DNA-dependent RNA polymerases. This observation supports the concept that the secondary structure of the RNA template plays an important role in the editing mechanism. Thomas *et al.* (1988) showed that the editing site of SV5 has a stem–loop structure which is supposed to be essential for the editing activity of the viral polymerase. The EBO GP gene sequence, like the SV5 P gene sequence, contains a stretch of nucleotides in the 5′ direction of the editing site which has the potential to form a hairpin-like secondary structure (Fig. 5). This secondary structure cannot be formed when DNA is used as template, which may explain the decreased editing efficiency observed under these conditions. Taken together, these observations indicate that the hairpin-like structure is supportive, but not essential for editing.

In conclusion our data suggest that expression systems using T7 polymerase or vaccinia virus polymerase are able to carry out frame shifting by RNA editing. Prerequisite for this editing, however, is a suitable editing site like that found inside the EBO GP gene. Further investigations are planned to study the influence of the sequence context around the editing site including the proposed secondary structure.

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